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Effect of pH on the Participation of Calcium Ion in the Cell Aggregation of Sea Urchin Embryos

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ABSTRACT—The effect of pH on the cell aggregation of sea urchin embryos was investigated to demonstrate the involvement of Ca^{2+} in cell aggregation. The cell aggregation was maximum at pH 8, decreased gradually at lower pHs and the aggregation did not occur below pH 4. On further examination, the pH profile of $^{45}Ca^{2+}$-binding to the aggregation factor was found to be almost identical with that of cell aggregation. The similarity of these pH profiles suggested the involvement of electrostatic interaction between Ca^{2+} and negatively charged groups of the aggregation factor in the cell aggregation of sea urtin embryos.

INTRODUCTION

The success in immunochemistry stimulated hypotheses involving antigen-antibody like reactions and sugar-lectin type reactions as the specific motive forces of cell aggregation. This line of research has advanced so far to propose the molecular mechanism for the interaction of aggregation factor and cell surface receptor [1–4]. On the other hand, biophysical approach has suggested the participation of electrostatic forces and van der Waals forces. Ca bridge hypothesis is most well known among them [5–7].

The cell aggregation factor of sea urchin embryos was shown to be a highly negatively charged sugar-protein complex extracted with Ca^{2+}-g, Mg^{2+}-free antifical sea water (CMF-SW). However, it has not yet been well characterized because of its instability [8]. The surface charge of dissociated cells is known to be negative at the pH of sea water [9]. Ca ion is indispensable for cell aggregation and the rate of cell aggregation is dependent on Ca^{2+} concentrations [8]. Hence it is reasonable to assume that the electrostatic forces may play some substantial role in the cell aggregation of sea urchin embryos.

Following experiments were designed to examine this possibility. The cell aggregation was significantly affected by pH with a maximum aggregation at pH 8. The binding of $^{45}Ca^{2+}$ to the aggregation factor showed almost the same pH profile as that of cell aggregation. These results are discussed with special reference to the mode of Ca^{2+} involvement in cell aggregation.

MATERIALS AND METHODS

A Japanese sea urchin, *Hemicentrotus pulcherrimus*, was mostly used for the following experiments.

Preparation of aggregation factor and hyaline layer substance

Aggregation factor was prepared from swimming blastula embryos following the method previously described [8]. Hatched blastulae were washed twice with Ca^{2+}-, Mg^{2+}-free artificial sea water (CMF-SW) and gently stirred in ice-chilled CMF-SW for 60 min until embryos were dissociated into constituent cells. After removing dissoci-

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ated cells with low-speed centrifugation (3000×g, 5 min), the suernatant was subjected to high-speed centrifugation (10,000×g, 20 min) at 4°C. The clear extract obtained was used as the cell aggregation factor.

To prepare [^{35}S]-labeled aggregation factor ([^{35}S]-AF), fertilized eggs were raised in [^{35}S]-$MgSO_4$-containing artifical sea water (5 μCi/200 ml). At blastula stage, metabolically labeled aggregation factor was extracted with CMF-SW according to the same method as that to prepare unlabeled eggregation factor.

Hyaline layer substance was extracted from fertilized eggs with CMF-SW and purified by precipitation with Ca^{2+} as was described previously [8].

Assay of cell aggregating activity

Dissociated blastula cells after the extraction of the aggregation factor were washed twice with CMF-SW, filtered through nylon mesh (380 mesh) and the cell numbers were counted with hemocytometer. Cell aggregation assay was performed on a zyratory shaker at 4°C. One ml of cell suspension (10^7 cells) and 1 ml of aggregation factor (100 μg protein) were added to a 30 ml Erlenmeyer flask containing 3 ml of Herbt's artificial sea water buffered with citrate (5 mM) for pH 3–6 or with Na-barbiturate (5 mM) for pH 7–9. After rotation (80 rpm, 10 mm rad.) for 60 min, the average number of cells in an aggregate was determined as was described before [8].

To change the pH of the medium in the course of experiment, 1 ml of 50 mM buffer of different pH was added to the flask after 60 min of rotation in the first medium. Rotation was continued for further 60 min and the cell aggregation was scored. To prepare the fixed cells, dissociated cells were fixed with cold glutaraldehyde (1% in CMF-SW, buffered to pH 8 with Tris-HCl for 3 hr and dialyzed thoroughly to remove excess glutaraldehyde. The fixed cells were filtered through nylon mesh (380 mesh) to remove small aggregates formed during fixation.

Preparation of cell surface glycopeptide

Dissociated blastula cells were treated with 0.1% trypsin in buffered CMF-SW (10 mM Tris-HCl, pH 8.0) at 20°C for 1 hour. After removing the cells by low speed centrifugation (1000×g, 5 min), the extract was spun at 10,000×g for 20 min and the supernatant was concentrated by ultrafiltration (Amicon PM-30). The concentrated trypsin extract was fractionated by gel filtration through Sephadex G-50 column (2×100 cm). The first fraction containing most of the sugar was pooled, lyophilized and used as cell surface glycopeptide.

Estimation of $^{45}Ca^{2+}$-binding

To estimate the binding of $^{45}Ca^{2+}$ to the cells, 1 ml of cell suspension (10^7 cells) and 1 ml of CMF-SW or aggregation factor (100 μg/ml) were added to 3 ml of buffered artificial sea water containing 2 μCi of [$^{45}Ca^{2+}$]-$CaCl_2$. After rotation for 30 min in the cold (80 rpm, 20 mm rad.), the cells were separated from the supernatant by centrifugation (1000×g, 5 min) and suspended in 5 ml of the same buffer. After standing for 10 min, the cells were spun down and transferred into scintillation vials, and the radioactivity was counted with a scintillation counter following addition of 10 ml of Triton-toluene scinillator. Additive washing of the cells did not affect the counting.

The binding of $^{45}Ca^{2+}$ to cell surface glycopeptide and to the aggregation factor was estimated by gel filtration. Small columns of Sephadex G-50 (6×400 mm) were equilibrated with buffered CMF-SW of different pHs. One hundred microliters of sample solution (cell surface glycopeptide, 100 μg; aggregation factor, 500 μg protein) were mixed with 100 μl of CMF-SW containing 1 μCi of $^{45}Ca^{2+}$. After incubation for 15 min at 0°C, the mixture was applied on the top of the column and eluted with the same buffer. Ten drop fractions were collected in each scintillation vial and the radioactivity was counted after addition of Triton-toluene scintillator. The peak appeared in the void volume was separated from the later peak retarded by the gel. The counts of the former peak were summed and estimated as bound $^{45}Ca^{2+}$ to macromolecular components.

Estimation of [^{35}S]-AF binding

The binding of [^{35}S]-AF to the cell was determined with the same procedure as that for $^{45}Ca^{2+}$

-binding, except for the use of [^{35}S]-AF (45,000 cpm/100 μg protein/ml) instead of $^{45}Ca^{2+}$.

RESULTS

Effect of pH on cell aggregation

The cell aggregation induced by the aggregation factor was examined in artificial sea water of various pHs. As is shown in Figure 1, cell aggregation was dependent significantly on pH and was maximal at pH 8. The sizes of cell aggregates decreased gradually at lower pHs and no aggregates formed below pH 4. Cell aggregation did not occur at any pHs in the absence of divalent cations and also without the aggregation factor.

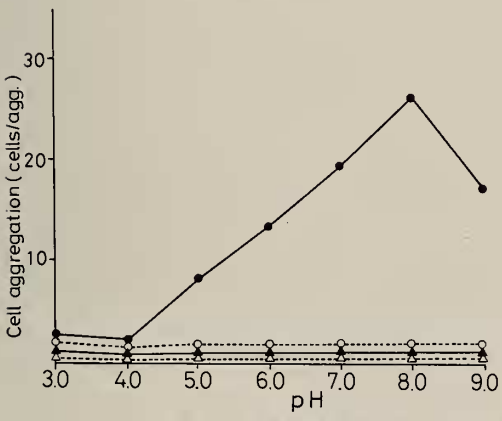

FIG. 1. Effect of pH on the cell aggregation of sea urchin embryos. Cell aggregation in the presence of the aggregation factor in artificial sea water (●—●), the same in CMF-SW (○---○); cell aggregation in the absence of the aggregation factor in artificial sea water (▲—▲), the same in CMF-SW (△---△).

A similar experiment was done with fixed cells to examine the possibility that the effect of pH on cell aggregation might be due to its effect on cell metabolism. The general pH profile of cell aggregation with fixed cells was similar to that of intact cells, although the extent of cell aggregation was markedly reduced (Fig. 2).

When pH was changed in the course of experiment (60 min) and the cells were kept at the second pH for further 60 min, the size of cell aggregates shifted close to that kept at the second

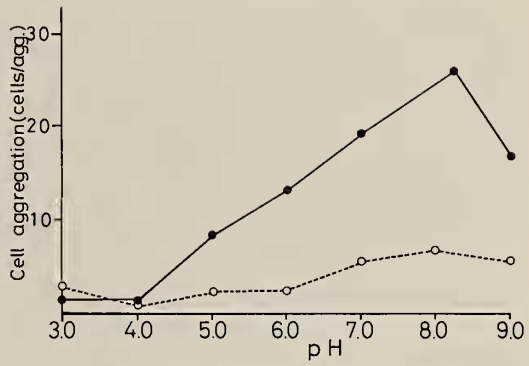

FIG. 2. Effect of pH on the aggregation of fixed cells of sea urchin embryos. Aggregation of fixed cells in the presence of the aggregation factor in artificial sea water (○---○) and aggregation of living cells under the same conditions (●—●).

pH from the beginning. When pH was changed from 4 to 8, the cells started to aggregate to reach the similar size to that of the aggregates formed on incubation at pH 8 (cells/aggregate, from 1.0 to 17.3). When pH was changed from 8 to 4, the cell aggregates began to dissociate but they were not completely dissociated after 60 min (cell/aggregate, from 24.5 to 11.6).

Effect of pH on the binding of ^{35}S-labeled aggregation factor to the cells

The binding of ^{35}S-labeled aggregation factor ([^{35}S]-AF) to the cells was examined in normal and CMF-SW (Fig. 3). The binding of [^{35}S]-AF in normal sea water was the highest at pH 8–9 and decreased as pH was lowered to 6, but increased again at pH 5–3. On the other hand, its binding in CMF-SW was low and did not change significantly from pH 9 to 5 but increased at pH 4–3. When the [^{35}S]-AF binding value in CMF-SW was subtracted from its binding value in normal sea water, the resulting pH profile (divalent cation-dependent binding) turned out to be similar to that of cell aggregation (Fig. 3).

Effect of pH on the binding of $^{45}Ca^{2+}$ to the cells and to cell surface glycopeptide

The binding of $^{45}Ca^{2+}$ to dissociated cells was examined in the absence and presence of the aggregation factor. The binding of $^{45}Ca^{2+}$ to the cells was the highest at pH 9 within the pH range

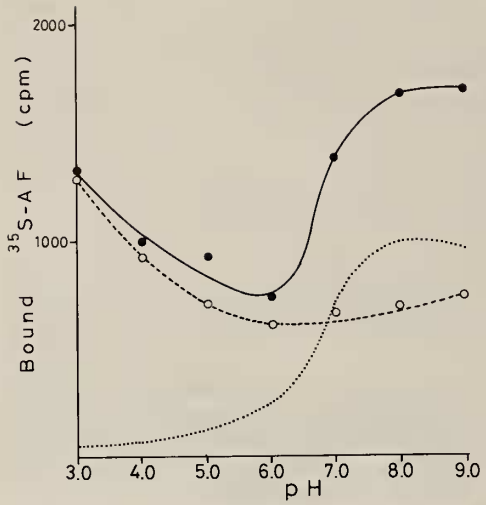

FIG. 3. Effect of pH on the binding of ^{35}S-labeled aggregation factor ([^{35}S]-AF) to the cells in artificial sea water (●—●) and in CMF-SW (○···○). Dotted line indicates the difference between the values.

examined and decreased continuously to pH 6 and retained half the maximal level at lower pHs (Fig. 4). The presence of aggregation factor at the concentration enough to cause cell aggregation did not alter this profile. In addition, the binding of $^{45}Ca^{2+}$ to cell surface glycopeptide showed a similar profile (Fig. 4).

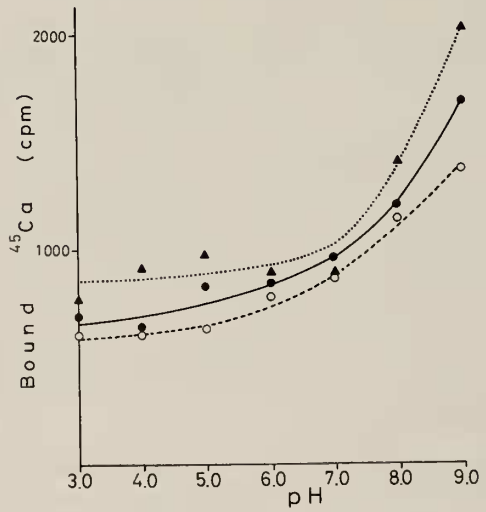

FIG. 4. Effect of pH on the binding of $^{45}Ca^{2+}$ to the sea urchin embryo cells in CMF-SW in the presence (▲···▲) and absence (●—●) of the aggregation factor, and the effect of pH on the binding of $^{45}Ca^{2+}$ to cell surface glycopeptide in CMF-SW (▲···▲).

Effect of pH on the binding of $^{45}Ca^{2+}$ to the aggregation factor

The binding of $^{45}Ca^{2+}$ to the aggregation factor was analyzed by gel filtration. $^{45}Ca^{2+}$ was bound to the aggregation factor maximally at pH 7–9, and its binding was also dependent on pH. It decreased continuously at lower pHs, and no significant binding occurred below pH 4 (Fig. 5). This pH profile of $^{45}Ca^{2+}$ binding to the aggregation factor was almost identical to that of cell aggregation and to that of divalent cation-dependent [^{35}S]-AF binding to the cells.

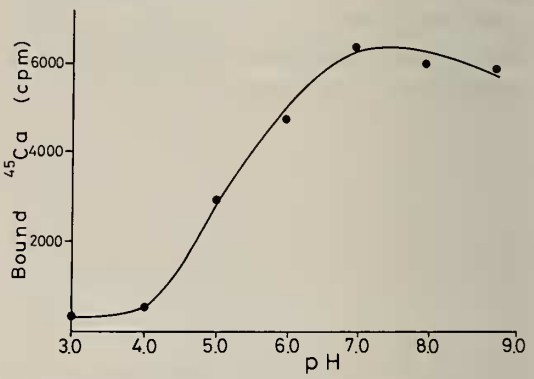

FIG. 5. Effect of pH on the binding of $^{45}Ca^{2+}$ to the aggregation factor of sea urchin embryos in CMF-SW.

Cell aggregation induced by the hyaline layer substance

The hyaline layer substance manifested cell aggregating activity at all pHs tested in the pre-

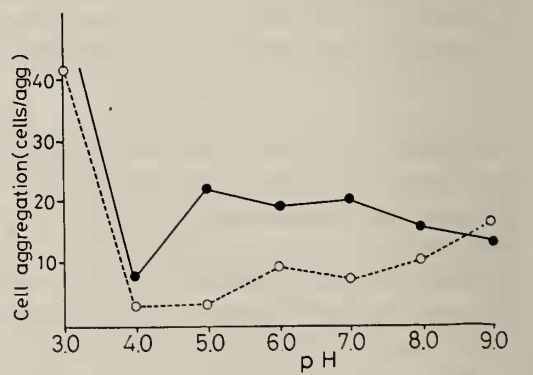

FIG. 6. Effect of pH on the cell aggregation induced by hyaline layer substance. Cell aggregation in the presence of hyaline layer substance in artificial sea water (●—●), the same in CMF-SW (○···○).

sence of Ca^{2+}. The rate of cell aggregation did not change significantly between pH 9 and 5. It was reduced at pH 4 but large cell clumps were formed at pH 3 (Fig. 6). Cell aggregating activity was also observed in the absence of divalent cations at hygher pHs. These pH profiles were quite different from those by the aggregation factor.

DISCUSSION

There has been a number of reports on the effects of pH on cell aggregation [7, 10, 11]. These authors showed pronounced cell aggregation at physiological pHs and reduced aggregation at lower pHs. In the present study, cell aggregation, induced by the aggregation factor of sea urchin embryos, was examined at a wide range of pHs and the extent of cell aggregation was shown to be remarkably influenced by pH. This pH dependency suggested the participation of electrostatic forces in cell aggregation. The change of pH would influence the ionization of charged groups of cell aggregation-related molecules and consequently the electrostatic interaction among them.

When one considers the necessity of Ca^{2+} in cell aggregation, it would be natural to take account of the electrostatic interactions among positively charged Ca^{2+} and negatively charged groups of the cell surface and those of the aggregation factor. These negatively charged groups, when not ionized at lower pHs, would not interact with Ca^{2+}. At higher pHs, on the contrary, they would be ionized and accordingly be ready to interact electrostatically with Ca^{2+}. Thus pH dependent ionization of the negatively charged groups appears to be the cause of pH dependent cell aggregation.

There is a possibility that the pH dependency of cell aggregation is due to indirect effect of pH through cell metabolism. However, cell aggregation with fixed cells showed almost a similar pH profile to that with live cells and the effect of pH on cell aggregation was reversible. These results favor the view that cell aggregation is influenced by pH through reversible ionization of the charged groups.

Previous experiments with labeled aggregation factor [12], have shown that [^{35}S]-AF bound to the cells and the binding was quantitatively proportional to the rate of cell aggregation. They suggested actual involvement of the aggregation factor in cell aggregation as an essential constituent. Accordingly, the effect of pH on [^{35}S]-AF binding to the cells was examined in the present experiments. Unexpectedly, its pH profile was not similar to that of cell aggregation, and a considerable extent of binding was detected at lower pHs. However, when the value of [^{35}S]-AF binding in CMF-SW was subtracted from that in normal sea water, the value of divalent cation-dependent binding manifested the same pH profile as that of cell aggregation.

When one assumes that the aggregation factor and Ca^{2+} constitute the intercellular bridges in cell aggregation, two sites are possible to be influenced by pH; one between Ca^{2+} and the negatively charged groups of the cell surface and the other between Ca^{2+} and those of the aggregation factor. The binding of $^{45}Ca^{2+}$ to the cells was shown to be independent of pH at lower pHs and the general profiles was quite different from that of cell aggregation. On the contrary, the pH profile of $^{45}Ca^{2+}$-binding to the aggregation factor was almost identical to that of cell aggregation. Therefore, it would be reasonable to conclude that the linkage between Ca^{2+} and the aggregation factor constitutes the rate limiting step in the effect of pH on cell aggregation. It is assumed that the binding of Ca^{2+} and the aggregation factor forms an essential link in the cell aggregation.

When one admit this assumption, the linkage of aggregation factor to the cell surface still remains to be elucidated. In our experiments, pH-dependent binding of [^{35}S]-AF to the cells needed Ca^{2+}, but it was also true that some significant portion of [^{35}S]-AF bound to the cells without Ca^{2+}. As was reported in other systems [13–16], it would be also possible that the aggregation factor binds the cell surface by another mechanism without the participation of Ca^{2+}. Calcium ion might just enhance the cooperation among the appregation factors to stabilize the intercellular bridges. Further studies to identify the mode of binding of the aggregation factor and to elucidate the mechanism of its binding to the cell surface are needed to settle this problem.

The pH profile of cell aggregation induced by the hyaline layer subtance turned out to be entirely different from that by the aggregation factor. Hyaline layer substance is a different cell aggregating agent from the so called aggregation factor [8]. Present result has added another proof to distinguish them.

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